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TITLE: A molecular basis accounted for the malignant features of breast cancer cells

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14. ABSTRACT

Malignancy is defined as the elevated mobility and invasiveness of tumor cells, and a deformed nuclear morphology is a common feature of malignant cells. We hypothesized that the decrease or absence of nesprin-1 in breast cancer cells may account for the malignant features of the neoplastic cells, the deformed nuclear morphology and invasiveness/high motility. We propose a pilot study to test this hypothesis. Indeed, we found that nesprin-1 expression is commonly lost in malignant breast cancer cell lines (Aim 1). We found that the suppression of nesprin-1 by siRNA led to nuclear morphological deformation and increased invasion (Aim 2). We also tested restoration of nesprin-1 expression in malignant breast cancer cells and nesprin-1 was not sufficiently stable to produce other significant phenotypes in the transfected cells, likely due to technical limitation (Aim 3). The results of these pilot experiments support the initial hypothesis of nesprin-1 as a metastatic suppressor gene and as an underlying link between two prominent features of a malignant cell, nuclear deformation and cellular malleability. We also realize further complexity of nesprin-1 function in breast cancer suppression. The pilot study promotes us to seek further investigation into the role of nesprin-1 in cancer malignancy.

15. SUBJECT TERMS
nuclear envelope, nesprin/SYNE, nuclear morphology, nuclear deformation, cellular malleability metastasis.

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Additional report cover the extension period   April 10, 2012
RE: BC097195 - "A Molecular Basis Accounted for the Malignant Features of Breast Cancer Cells"

Title: **A Molecular Basis Accounted for the Malignant Features of Breast Cancer Cells**

Xiangxi Michael Xu, Ph.D.

We have been granted a period of no cost extension, from the original period of 15-SEP-2010 to 14-OCT-2011, to the new project period: 15-SEP-2010 to 14-JAN-2012. The three month extension is to make additional attempt to work on the not yet completed tasks:

Task 3 (month 3): Perform qRT-PCR for nesprin-1 in total RNA isolated from 50 frozen breast cancers, with at the least 3 non-cancer controls. We will be able to determine if loss of nesprin-1 correlates with tumor grades and degree of malignancy in breast cancer tissues.

Task 4 (month 3): Collect around 100 breast cancer samples in tissue microarray. Perform immunostaining for nesprin-1. We will be able to determine if loss of nesprin-1 correlates with invasion and metastasis in breast cancer tissues.

For Task 3, we have tested and established a RT-PCR protocol to measure the expression of several nesprin-1 isoform. Effort is still been made to obtain frozen breast tumor tissues for analysis. Our institutional tumor bank has such tissues, but in three samples obtained, we found that quality of RNA is not sufficient for a proper measurement. Thus, we decided that it is not feasible to complete this task in the near future, and we will need to improve the protocol of preserving tumor tissues in the tumor bank. However, the information of nesprin-1 expression can be obtained by immunostaining.

For Task 4, in the last three months we have made and tested two mouse monoclonal (to two different peptides) and one rabbit polyclonal antibodies to nesprin-1. The rabbit antibodies were found to be able to recognized over-expressed protein, but the anti-serum do not have sufficient specificity and affinity to recognize the endogenous protein. So far, we have tested three clones of monoclonal antibodies (positive in peptide binding assay), but none have not yet been able to recognize the nesprin-1 protein. We will do additional screening of additional monoclonal antibody clones. However, we cannot complete Task 4 currently. However, we will make additional effort and hope to complete the task of assaying nesprin-1 expression in breast tumor tissues in the future.

In sum, despite of additional effort in the last three months, we have not yet completed Task 3 and 4. We hope to do more work in future project to obtain the information of nesprin-1 expression in tumor tissues. Thus, the work from the period of extension has not changes the outcome of the project, as described in the final report followed.

Table of Contents

INTRODUCTION: Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

The severity of cancer, known as the degree of malignancy, is caused by the ability of the cancer cells to move and invade tissues (malleability). Unlike normal cells which have a smooth and oval shape nucleus, the cancer cells often have deformed, irregular nuclear shape, and this phenomenon is used to diagnose the degree of malignancy, known as nuclear grade (1,2). In this project, we investigated an idea, that the two features of cancer cells, deformed nuclear shape and high mobility, may be caused by the loss of nesprin-1, a structural protein linking nuclear envelope to the cytoskeleton of cells (3-5). The pilot experiments are to study nesprin-1/Syne-1 as a metastatic/invasion suppressor gene and as an underlying link between two prominent features of a malignant cell, nuclear deformation and cellular malleability.

BODY: This section of the report shall describe the research accomplishments associated with each task outlined in the approved Statement of Work. Data presentation shall be comprehensive in providing a complete record of the research findings for the period of the report. Provide data explaining the relationship of the most recent findings with that of previously reported findings. Appended publications and/or presentations may be substituted for detailed descriptions of methodology but must be referenced in the body of the report. If applicable, for each task outlined in the Statement of Work, reference appended publications and/or presentations for details of result findings and tables and/or figures. The report shall include negative as well as positive findings. Include problems in accomplishing any of the tasks. Statistical tests of significance shall be applied to all data whenever possible. Figures and graphs referenced in the text may be embedded in the text or appended. Figures and graphs can also be referenced in the text and appended to a publication. Recommended changes or future work to better address the research topic may also be included, although changes to the original Statement of Work must be approved by the Army Contracting Officer Representative. This approval must be obtained prior to initiating any change to the original Statement of Work.

In the last year, we have followed the research plan described in the Statement of Work (SOW). We have accomplished most of the experiments proposed and reached the conclusion to support our hypothesis that loss of nuclear-cytoplasmic linker protein nesprin-1 in breast cancer cells may account for the malignant features of the neoplastic cells, the deformed nuclear morphology and invasiveness/high motility, and the loss of nesprin-1 may account for the increased cellular malleability and invasive behavior of the cancer cells.

A series of tasks were undertaken to accomplish the 3 aims in this proposal with predetermined milestones, and the results are described under each milestone.

Aim 1. We will determine/confirm if nesprin-1 expression is commonly lost in malignant breast cancer tissues and cell lines, compared to and correlated with benign tumors and primary human breast cancer cells.

Aim 2. We will determine if suppression (by siRNA and shRNA) of nesprin-1 in primary breast epithelial cells and non-metastatic breast cancer cells affects cell growth, leads to nuclear morphological deformation, and increases mobility and metastatic potential.

Aim 3. We will test if restoration of nesprin-1 expression (by cDNA transfection) in malignant breast cancer cells affects cell growth and suppress mobility and metastatic potential.

Task 1 (month 1-2): Collect 15 breast cancer cell lines and 3 non-cancer primary human mammary epithelial cell lines. Grow and expand these cells in cell culture, isolate cell lysate and mRNA for further analysis.

Task 2 (month 2): Use the mRNA and cell lysates prepared to perform qRT-PCR and Western blot to measure expression of nesprin-1. Multiple antibodies against the C- and N-terminal portion of nesprin-1 will be used to detect several spliced and alternative promoter isoforms. We will find out if nesprin-1 is commonly lost in breast cancer cells compared to non-cancer mammary epithelial cells, and if the loss of nesprin-1 correlates with malignancy of the cells.

Task 3 (month 3): Perform qRT-PCR for nesprin-1 in total RNA isolated from 50 frozen breast cancers, with at the least 3 non-cancer controls. We will be able to determine if loss of nesprin-1 correlates with tumor grades and degree of malignancy in breast cancer tissues.

Task 4 (month 3): Collect around 100 breast cancer samples in tissue microarray. Perform immunostaining for nesprin-1. We will be able to determine if loss of nesprin-1 correlates with invasion and metastasis in breast cancer tissues.

Milestone 1: Tasks 1-4 (Aim 1) will enable us to verify if nesprin-1 is commonly lost in breast cancer cells, and if the loss of nesprin-1 correlates with invasion and metastasis in breast cancer.

We speculated and planned to determine/confirm if nesprin-1 expression is commonly lost in malignant breast cancer tissues and cell lines, compared to and correlated with benign tumors and primary human breast cancer cells. Indeed, we found that nesprin-1 expression is commonly lost in malignant breast cancer tissues and cell lines **(Figure 1)**. We used a new technology, the nano-string approach (6) to determine the expression of nesprin-1 mRNA in breast cancer cells comparing to non-cancer cells, and found that nesprin-1 mRNA level is low or absent in all breast cancer cells analyzed, and a much higher level of expression is present in non-cancer human mammary epithelial cells.

Figure 1. Loss of the expression of Nesprin-1 (SYNE-1) in breast cancer cells. The mRNA expression of nesprin1 was determined by nanoString technology (6)in cells. The probe for nesprin-1 locates in the c-terminal KASH domain of human nesprin-1 gene. Five lines of primary cultures of human breast epithelial cells, a non-tumorigenic breast epithelial cell line (MCF-10), 4 breast cancer cell lines (MCF-7, MDAMB231, MDAMB468, and T47D), and 6 additional prostatic and endometrial cancer cell lines were analyzed. Generally, nesprin-1 expression is absent or greatly reduced in most cancer lines comparing to the non-tumorigenic breast epithelial cell line.
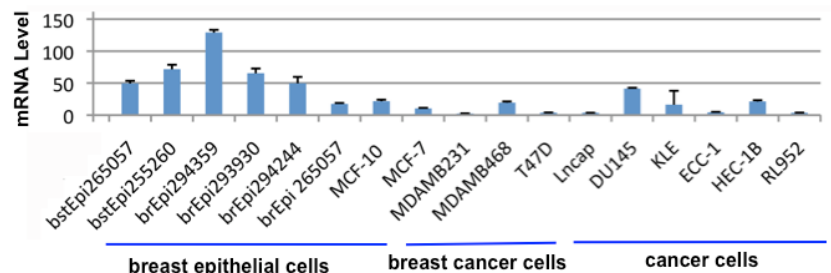
We purchased and tested 4 commercially available antibodies to nesprin-1 for Western blot and immunostaining. However, we have not been able to demonstrate that the antibodies recognized a nesprin-1 protein species or provided true staining in immunofluorescence microscopy. We concluded that these antibodies are not of sufficiently good quality for our experiments. We are currently in the process of making several monoclonal antibodies to peptides of human and mouse nesprin-1 sequences. Although these antibodies will not available yet, they will be useful for future studies based on this pilot project. These tasks have enabled us to conclude that nesprin-1 expression is lost or greatly reduced in breast and other cancer.

Task 5 (month 4-6): siRNA will be prepared to target nesprin-1, and at the least two effective sequences will be used. A panel of breast cancer cells (MCF-7, T47D) identified in Aim 1 that have high nesprin-1 expression, will be transfected with siRNA to suppress nesprin-1 expression. Three lines of primary human mammary epithelial cells will be also tested. The transfected cells will be analyzed for suppression of nesprin-1 expression by Western blot.
Task 6 (month 6): The siRNA transfected cells will be analyzed for nuclear morphology by immunofluorescence microscopy, to observe the potential deformed cancer cell nuclear morphology following nesprin-1 suppression. The cells will be analyzed (comparing to scrambled siRNA controls) for growth, mobility, Boyden chamber invasion, and xenograft in nude mice to determine tumorigenesis and metastasis.

Milestone 2: These experiments in Task 5 and 6 (Aim 2) will determine/confirm if down regulation of nesprin-1 is sufficient to induce deformed nuclear morphology and increased mobility/invasion of the non-cancer mammary epithelial cells and non-metastatic cancer cells.

From the experimental results in **Aim 1**, we determined that the majority of breast cancer cell lines have low nesprin-1 expression, thus, no suitable cancer cell lines can be used for suppression of nesprin-1. When the

expression of nesprin-1 was suppressed by siRNA and shRNA in primary breast epithelial cells (**Aim 2**), we found that the suppression led to nuclear morphological deformation, though we have not been able to sufficiently analyze cell growth and metastatic potential due to technical reason. The suppression of nesprin-1 is not sufficiently effective and long lasting for proper measurement of cell growth and mobility. The relatively short lifespan of primary human breast epithelial cells makes such experiments difficult.

In order to test the idea in this aim and to reach milestone, we used an available ovarian surface epithelial cell preparation available to us in the lab. The cells were prepared from a p53 null mouse and can grow well in tissue culture for extensive period. We transfected siRNA vector to target nesprin-1 sequence. The cells were implanted into nude mice to test tumorigenesis and tumor histology. In 3 out of 4 nude mice injected, tumors developed following 4 months, indicating a prolong latency. The tumors in all three mice exhibit similar histology: tumor cells are distributed in small nodules and intermingled with muscle fibers and stroma (**Figure 2**). The histology suggests that nesprin-1 suppressed cells are highly mobile and invasive. Thus, basically, the preliminary data is supportive of the hypothesis that loss of nesprin-1 may promote tumor cell mobility and invasion. The experiments enable us reaching **Milestone 2**.

Figure 2. Loss of Nesprin-1 (SYNE-1) expression promotes tumor cell mobility and invasiveness. The representative H&E images of tumor developed in nude mice from nesprin-1 suppressed cells are shown in lower magnification (left panel) and high magnification (right panel, of the boxed area).
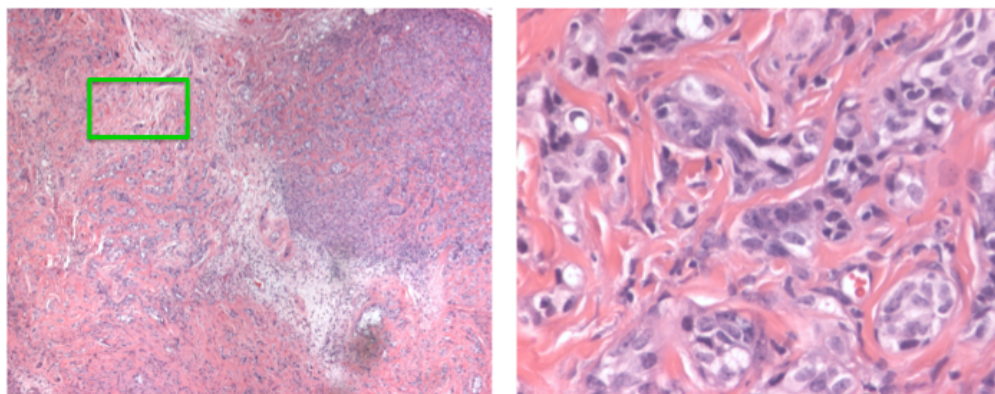
Task 7 (month 4-8): In 3 lines of nesprin-1-negative (determined in Aim 1) metastatic breast cancer cells, nesprin-1 will be transfected and expressed. The transfected clones will be selected by neo-resistance and the clones will be expanded.
Task 8 (month 9-11): The nesprin-1 expressing cells will be assayed for growth, mobility, invasion, and tumorigenesis/metastasis in nude mice, comparing to parental lines.

Milestone 3: Task 7-8 will accomplish Aim 3: To verify if nesprin-1 is a metastatic suppressor gene.

We also tested if restoration of nesprin-1 expression (by cDNA transfection) in malignant breast cancer cells affects cell growth and suppress mobility and metastatic potential (**Aim 3**). Since we are not able to monitor the expression of nesprin-1 definitely because of lacking a suitable antibody, we used a mCherry fusion to visualize nesprin-1 expression. Nesprin-1 gene produces multiple protein isoforms (**Figure 3**), we tested the 125 kd KASH domain containing isoform in our study. Expression of mCherry-nesprin-1 in T47D cells shows localization of the protein as a ring around nucleus as well as distribute in cytoplasm (**Figure 4**). Re-expression of mCherry-nesprin-1 was found to alter nuclear envelope morphology. However, the expressed nesprin-1 is not sufficiently stable to produce other significant phenotypes in the transfected cells.

Figure 3. The structural isoforms of Neprin-1/Syne1. illustrated. The several known SYNE1/nesprin-1 protein isoforms derived from the gene are illustrated to indicate the actin binding calponin domain, KASH domain, and the Spectrin repeat rod domain (3). The 120 kd isoform of nesprin-1 was fused with mCherry and used for transfection into breast cancer cells.
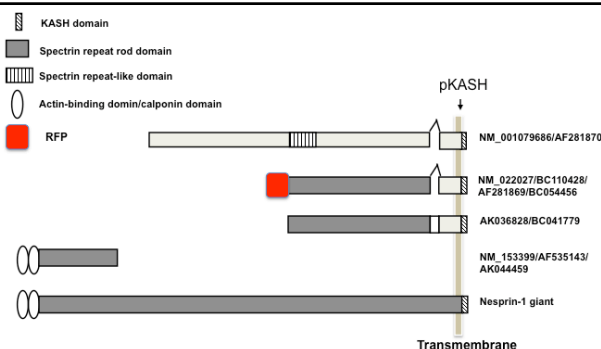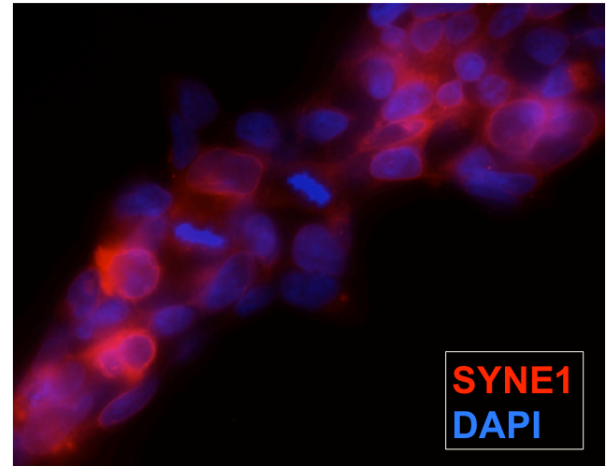
Figure 4. Lamin A expression in T47D breast cancer cells: immunofluorescence microscopy. T47D breast cancer cells were transfected and selected for mCherry-nesprin-1 expression. The expression of mCherry-nesprin-1 (SYNE1) (red) was visualized by fluorescence microscope. DAPI staining (blue) was used to visualized nucleus.

Task 10 (month 12-14): Prepare final report to DOD. Prepare manuscript to report the potential findings!
Milestone 4: We will be able to conclude whether nesprin-1 is a breast cancer metastatic suppressor gene, and whether loss of nesprin-1 accounts for both nuclear deformation and cellular malleability of malignant breast cancer cells.

The results of these pilot experiments support the initial hypothesis of nesprin-1 as a metastatic suppressor gene and as an underlying link between two prominent features of a malignant cell, nuclear deformation and cellular malleability. The **Milestone 4**, obtaining evidence to support a metastasis suppressor role of nesprin-1, has been basically achieved. We also realize further complexity of nesprin-1 function in breast cancer suppression. The pilot study provides basis and promotes us to seek further investigation into the role of nesprin-1 in cancer malignancy.

KEY RESEARCH ACCOMPLISHMENTS:

This research project has been completed to satisfactory and enabled us to obtain supportive information for the overall hypothesis that nesprin-1 is lost in breast cancer and this may promote tumor cell invasion. The results are preliminary, though we are able to reach the following 3 conclusions.

1. We found that nesprin-1 expression is commonly lost in malignant breast cancer tissues and cell lines.
2. When the expression of nesprin-1 was suppressed by siRNA and shRNA in primary breast epithelial cells, we found that the suppression led to nuclear morphological deformation.
3. We found that suppression of nesprin-1 expression does not significantly influence cell proliferation, but enhanced metastasis.

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research to include:
- manuscripts, abstracts, presentations; patents and licenses applied for and/or issued; degrees obtained that are supported by this award; development of cell lines, tissue or serum repositories; infomatics such as databases and animal models, etc.; funding applied for based on work supported by this award; employment or research opportunities applied for and/or received based on experience/training supported by this award

The concept award allowed us to make initial study of our hypothesis. The preliminary results support the hypothesis that a defective nuclear envelope structure, due to the loss of nuclear envelope structure protein nesprin-1/SYNE-1, may be the underlying mechanism for nuclear morphological deformation and mobility and invasiveness of malignant breast cancer cells. The pilot results will be used to support application of additional fund to continue

CONCLUSION: Summarize the results to include the importance and/or implications of the completed research and when necessary, recommend changes on future work to better address the problem. A "so what section" which evaluates the knowledge as a scientific or medical product shall also be included in the conclusion of the report.

The results of these pilot experiments support the initial hypothesis of nesprin-1 as a metastatic suppressor gene and as an underlying link between two prominent features of a malignant cell, nuclear deformation and cellular malleability. We also realize further complexity of nesprin-1 function in breast cancer suppression. The pilot study promotes us to seek further investigation into the role of nesprin-1 in cancer malignancy.

REFERENCES: List all references pertinent to the report using a standard journal format (i.e. format used in *Science, Military Medicine*, etc.).

1. Pienta KJ, Coffey DS. Correlation of nuclear morphometry with progression of breast cancer. Cancer 1991; 68:2012-2016.

2. Zink D, Fischer AH, Nickerson JA. Nuclear structure in cancer cells. Nat Rev Cancer 2004; 4:677-687.

3. Warren DT, Zhang Q, Weissberg PL, Shanahan CM. Nesprins: intracellular scaffolds that maintain cell architecture and coordinate cell function? Expert Rev Mol Med 2005;7(11):1-15.

4. Crisp M, Liu Q, Roux K, Rattner JB, Shanahan C, Burke B, Stahl PD, Hodzic D. Coupling of the nucleus and cytoplasm: role of the LINC complex. J Cell Biol 2006;172(1):41-53.

5. Olins AL, Hoang TV, Zwerger M, Herrmann H, Zentgraf H, Noegel AA, Karakesisoglou I, Hodzic D, Olins DE. The LINC-less granulocyte nucleus. Eur J Cell Biol 2009;88:203-214.

6. Geiss GK, Bumgarner RE, Birditt B, Dahl T, Dowidar N, Dunaway DL, Fell HP, Ferree S, George RD, Grogan T, et al.: Direct multiplexed measurement of gene expression with color-coded probe pairs. Nat Biotechnol 2008, 26:317-325.

APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

None

SUPPORTING DATA: All figures and/or tables shall include legends and be clearly marked with figure/table numbers.

None